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Use of Multivariate Analysis to Assess the Nutritional Condition of Fish Larvae From Nucleic Acids and Protein Content

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Abstract. The nutritional condition of turbot larvae (*Scophthalmus maximus*) was assessed by a multivariate analysis with DNA, RNA, and protein content as input variables. Special attention was given to the time that feeding began and to the timing and duration of starvation. The combination of the principal components analysis and the stepwise discriminant analysis, both techniques of multivariate analysis, made it possible to allocate the larvae into groups that were defined and identified based on similarities in developmental stage and nutritional condition. The developmental stage was mostly determined by the input variables DNA and protein content, while nutritional condition was determined by the RNA content. In the period studied, the more developed larvae were less resistant to starvation. Furthermore, when initial feeding was delayed as little as 6 h, the variables analyzed were markedly changed, and the effect on the deprived larvae was found to be equivalent to a 3-day delay in development—when compared with the larvae fed immediately after mouth opening. Through this technique, new samples of larvae with unknown history might be classified into groups, using their DNA, RNA, and protein content as input values in the defined classification functions. Results were compared to those obtained using RNA/DNA and RNA/dry weight indices, and the multivariate method was considered to be more sensitive and to provide extra information about larval nutritional history and development.

Introduction

Some events in the early life of fishes are critical to the fluctuations of fish populations in marine environments (Trippel and Chambers, 1995). Among those, larval mortality is widely acknowledged to be one of the most important (Cushing, 1975). Starvation and inadequate food supply are significant causes of mortality (Hjort, 1914; Cushing, 1974, 1975; Lasker, 1975). Feeding studies require that accurate and quantitative criteria be used to characterize the nutritional condition of fish larvae. This approach can also be applied in aquaculture (Quantz, 1989; Planas *et al.*, 1991) to interpret the adequacy of different diets for fulfilling the nutritional requirements of the cultured species and to establish the limits for starting exogenous feeding.

A variety of methods have been used to diagnose the nutritional condition and recent growth rate of fish larvae at different organizational levels: organism, tissue, and cellular. Methods based on biochemical criteria have been considered more effective and sensitive, because changes due to a feeding regime are first reflected at subcellular and cellular levels, and only thereafter in the whole organism (Robinson and Ware, 1988). RNA, DNA, and protein contents of the larvae have been used frequently as biochemical criteria (Bulow, 1970; Buckley, 1979 a,b, 1982, 1984; McGurk, 1984; Clemmesen, 1987, 1994; Buckley *et al.*, 1990, 1991; Bergeron *et al.*, 1991; Cunha, 1991, 1996; Richard *et al.*, 1991; Mathers *et al.*, 1992, 1993; Canino, 1994; Takii *et al.*, 1994; Clemmesen *et al.*, 1997; Gronkjaer *et al.*, 1997; Chícharo *et al.*, 1998; Bergeron, 2000). However, none of these variables alone can indicate a larva's nutritional condition, growth potential, or ability to recover from starvation. Instead, ratios that combine these variables in pairs are generally used (*e.g.*, Clemmensen *et al.*, 1997; Chícharo *et al.*, 1998).

However, the use of ratios is still a univariate technique that may provide only a quantitative description of the variation of such ratios among individuals. (If a single observation or measurement is made for each individual, the

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data are said to be univariate; more than one observation or measurement for each individual produces multivariate data (Krzanowski and Marriott, 1994).) Organisms are integrated units whose variables are intercorrelated to varying degrees. By not taking the interdependency of variables into account, many univariate analyses will overestimate the dimensionality of divergence (Atchley and Bryant, 1975). Hence, these variables should not be analyzed separately; instead the correct approach must be multivariate. Multivariate analysis is the only meaningful technique that examines the relationships among several variables, which helps to determine affinities between individuals and considers the variation in such variables as a whole—that is, allows exploration of variation in a multidimensional scale (Morrison, 1967). In our opinion, nutritional condition, which is the result of the interaction of more than two variables, is better studied through multivariate techniques.

Multivariate analysis has been used extensively in ecological studies (see: Digby and Kempton, 1987; Saila and Martin, 1987; Rodriguez and Magnan, 1995), primarily to analyze morphometric characters of both adult (Reyment *et al.*, 1984; Saborido-Rey, 1994) and larval fish (Theilacker, 1978, 1981, 1986; Powell and Chester, 1985; Polo, 1991; Reichow *et al.*, 1991). It has seldom, and only superficially, been applied to data on the biochemical composition of fish larvae (McGurk and Kusser, 1992; Navarro *et al.*, 1995; Chícharo, 1996).

An interesting feature of multivariate analysis, when used in studies of nutrition, growth, and development, is that it opens the possibility of assessing wild larvae, where the background of nutrition and development is unknown. However, as a first step, a set of experiments with cultured larvae is required to test the statistical methods and establish the models that will classify wild larvae.

In this study, the nutritional condition of cultured turbot larvae was assessed through a multivariate approach using the DNA, RNA, and protein content of larval tissues as variables. Turbot was the preferred species because of its importance in aquaculture and the depth of knowledge available about its biology, especially in relation to its feeding activities (Cunha and Planas, 1995; Cunha, 1996).

Material and Methods

Experimental conditions and sampling

Turbot larvae were obtained from a commercial hatchery where eggs were incubated at 14 °C. At 50% hatching, larvae were transported to the laboratory and stocked in two 250-l tanks (A and B). The temperature was slowly raised over the following 2 days from 14°C to 20–21°C. This temperature was maintained from larval mouth opening (day 3; $t = 0$), when sampling started, until the end of the experimental period.

Treatment A. Larvae in the tank were not fed. After mouth opening, groups of 1500 larvae were transferred after 6, 12, 30, and 54 h of starvation from the tank to 16-l buckets, where food was supplied, and reared according to Cunha and Planas (1995). Food density in the buckets was maintained at 5 items per milliliter, which guarantees an *ad libitum* supply of food. Samples were taken every 24 h until no more larvae were left, *i.e.* about 6.5 d later. Samples from the tank, where the larvae were always starved, were also taken at regular intervals and considered as the control for the starved larvae. The dry weight and protein, DNA, and RNA contents of these samples were determined. Treatment A in the Appendix summarizes the sampling regime and initial conditions for these larvae.

Treatment B. Larvae in this tank were fed *ad libitum* using the same standard technique. After first feeding (day 3), groups of 1500 larvae were transferred after 6, 30, 50, 96, and 150 h from the tank to 15-l buckets, where no food was supplied. These larvae were sampled at 6, 12, 24, 48, and 72 h after the onset of the starvation period, until 50% mortality was reached, *i.e.* about 3 days of food deprivation. Samples from the tank, where the larvae were always fed, were also taken at regular intervals and considered as the control for the fed larvae. The dry weight and protein, DNA, and RNA contents of these samples was determined as in Treatment A. Treatment B in the Appendix summarizes the sampling regime and the initial conditions for these larvae.

Biochemical analyses

Protein content was analyzed according to Lowry *et al.* (1951). Nucleic acids (DNA and RNA) were extracted and separated according to the technique developed by Schmidt and Thannhauser (1945) and modified by Fleck and Munro (1962), Fleck and Begg (1965), and Munro and Fleck (1966, 1969). The larvae for each sample were pooled to ensure that the analyzed biomass would be larger than 800 μg (Buckley, 1979a). Each pooled sample was referred to as a case; 61 cases were analyzed (Appendix).

Data analyses

Content of protein, DNA, and RNA was used to assess larval nutritional condition by a multivariate approach, combining principal components analysis and stepwise discriminant analysis. Before multivariate analysis can be done, it is important to screen the data for outliers and check the normality. However, multivariate analysis is considered robust enough to be insensitive to minor deviations in normality and homoscedasticity (Kirk, 1982; Harris, 1985). Outliers were detected by box plots; the normality of each variable was checked with the Shapiro-Wilks' test.

Principal components analysis (PCA) was performed on both sets of data taken together (Treatments A and B) and assumes no structure for the data. PCA studies the intercorrelations of a number of variables by clustering them into common factors, such that the variables within each factor are highly correlated, but the factors are uncorrelated.

Therefore, each of the factors explains a different part of the total variance in the data. The factors should be treated as new variables; and, therefore, each case has a value on the derived factors. These values are called factor scores. The PCA provides factor scores for each case, which can be plotted and investigated for the existence of any structure in the data, defining groups of cases with similar characteristics.

These groups were defined based not only on their factorial scores but also on the common and differential attributes of each case, that is, their nutritional condition and larval development. Thus, the groups were delimited using a biological definition. Nutritional condition was evaluated by considering the relative time that larvae were fed, or starved. Larval development was assessed by the size and age of the larvae; the age was expressed in *day degrees*. Age in days is not necessarily coupled with development, because larval development is very dependent on temperature. A day degree is a unit that combines temperature and time, and is useful for monitoring and comparing growth when temperatures fluctuate.

Although PCA assumes no structure in the data and the main goal is to sort cases (the so-called ordination technique), stepwise discriminant analysis (SDA) requires the existence of groups and tells us how different these groups are (discrimination or classification technique) by means of a *discriminant function*. The discriminant function is a linear combination of the original variables that best separate groups; therefore, a coefficient for each variable and a constant is calculated. SDA results in k-1 discriminant functions, k being the number of groups analyzed. The importance of each discriminant function in the analysis is given by its canonical correlation and the total variance explained by each function. Wilks' λ was used to assess the discriminatory effectiveness of the analysis (Wilks, 1932). This is a multivariate analysis of variance statistic that tests the equality of group means for the variables in the discriminant function. The smaller the λ, which ranges from 0 to 1, the greater the difference between groups.

On the other hand, SDA also results in *classification functions*, which are computed for each group and can be used directly to classify cases. A case will be classified into the group for which it has the highest classification score. It results in a classification matrix where the efficiency of the analysis can be also evaluated. Further description of multivariate techniques can be found in Hair *et al.* (1998).

The ratios RNA/DNA and DNA/dry weight were determined for each case, and mean values were calculated for each of the groups established by the multivariate analysis. The mean values of the groups were compared through ANOVA. The *post hoc* Tukey's honest significant difference (HSD) test for unequal n was used, since the groups obtained were rather unequal in size.

Statistica for Windows 5.0 (StatSoft Inc., 1995) was used to perform all the statistical analyses.

Results

The original data of protein, RNA, and DNA concentrations included in the statistical analysis for Treatments A and B are given in the Appendix. Dry weight and age (in day degrees) of the larvae are also shown in the table, as well as RNA/DNA and DNA/dry weight indices.

The principal components analysis extracted two factors, PC_1 and PC_2, that explained 99.7% of data variation, 50% each (Table 1). The most determinant variables for PC_1 were larval protein and DNA content, whereas for PC_2 the most determinant variable was RNA content. The graphical representation of the factor scores for each case, computed from loading rotated factors of PC_1 and PC_2, is presented in Figure 1. Based on examination of this plot, cases can be grouped according to the relative positions of their factor scores. At least four groups can be initially defined. To define these groups we also examined two criteria for each case: nutritional background and larval development. Nutritional condition was evaluated considering the relative time that larvae were fed or starved, and larval development was assessed by the size and age of the larvae. This examination revealed that the groups resulting from the PCA are well delimited in relation to these two criteria; thus a clear link is established between the biochemical analysis and the nutritional condition and developmental stage of the larvae. We named these groups G_1, G_2, G_3, and G_4. PC_1 allows the separation of G_2-G_3 from G_1-G_4, while PC_2 splits G_1-G_2 from G_3-G_4 (Fig. 1). Thus the combination of both factors, PC_1 and PC_2, allows the identification of the four groups.

Nevertheless, a continuous gradient was observed in the distribution of the cases on the factorial plot (Fig. 1). This sounds logical, taking into account that larvae used in the analysis not only were those in extreme conditions but also were sampled regularly throughout the experimental period. Thus an intermediate group was also found, distributed among groups G_1, G_2, and G_4, as shown in Figure 1, encircled by a dashed curve. To ascertain the distribution of these intermediate samples among the initial four groups, three different stepwise discriminant analyses were performed: (1) using only the four most distant groups; (2) incorporating the intermediate set of cases as a fifth group; and (3) with a new group definition, using all 61 cases.

Table 1

Standard coefficients of principal components (PC_1 and PC_2) obtained through principal components analysis

Variable	PC_1	PC_2
Protein	0.715	0.695
RNA	0.541	0.840
DNA	0.842	0.538
Variance explained (%)	50.5	49.2

Values show the relative importance of each variable in each principal component. Each PC explains approximately 50% of the total variance.

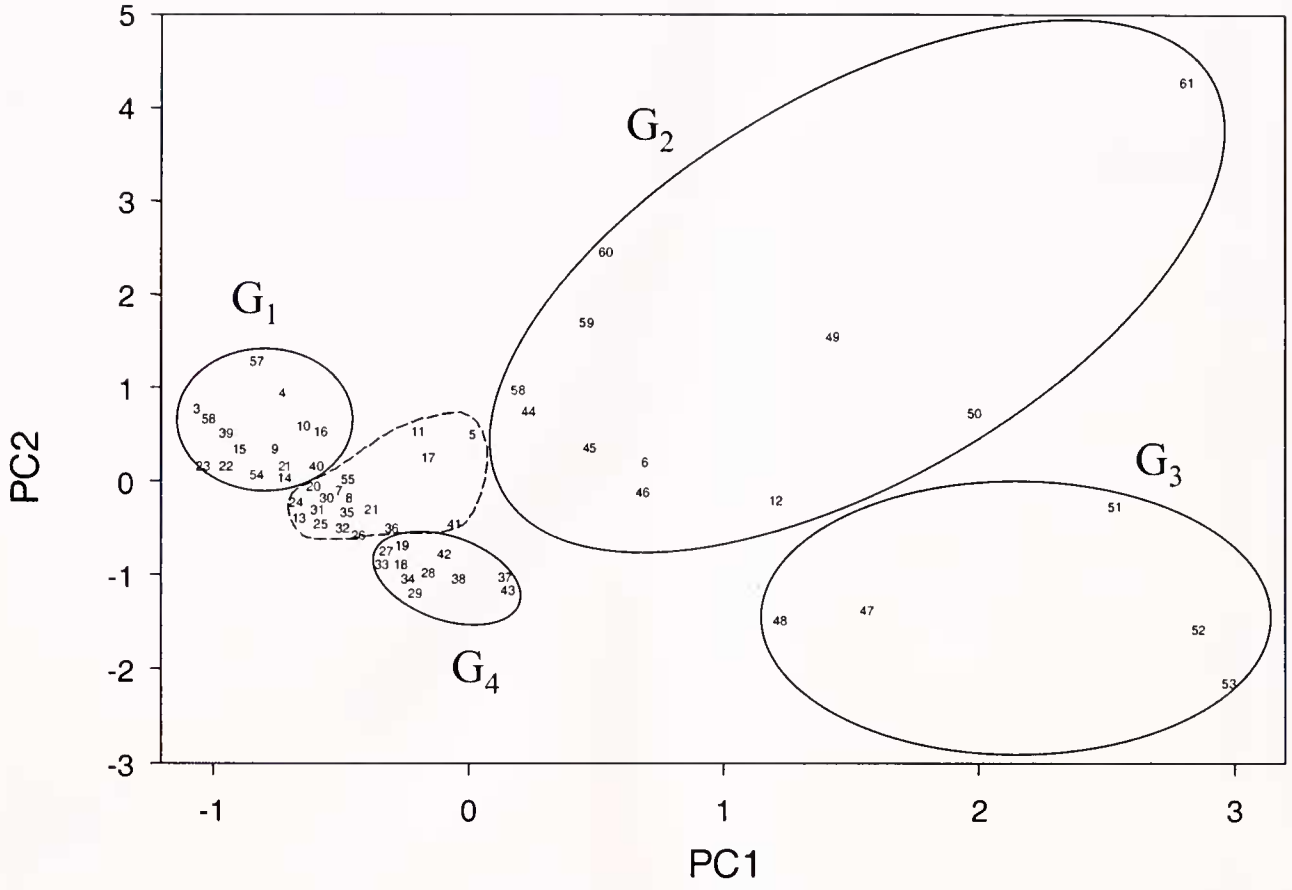

Figure 1. Plot of factor scores of the cases after principal components analysis with respect to first (PC_1) and second (PC_2) principal components. The distribution of the cases was compared with the larval development and the nutritional condition of each case to allow the definition of groups within the plot. Thus four initial groups (continuous line) and an intermediate group (dashed line) were defined.

The results of the first SDA evidenced good discrimination between the four groups. Wilks' λ was very low (0.038), and the final correct classification was high (97.67%). All the cases of groups G_1, G_3, and G_4 were correctly classified within their own groups (*i.e.*, 100% correct classification); and only one case of G_2 was misclassified as belonging to G_4 (90.91% correct classification). Thus, only 1 of 43 cases was misclassified. Therefore, the groups were considered to be distinct and the initial group definition was deemed appropriate.

In the next step, the intermediate group was incorporated into another SDA analysis. The results of this analysis showed a high degree of overlapping between G_1 and the intermediate group. Thus, 22.2% (4 of 18) of the intermediate group cases were classified as G_1; on the other hand, 12.5% (2 of 16) of G_1 cases were classified in the intermediate group. Of the intermediate group cases, 72.2% were correctly classified, as belonging to that group, indicating some degree of independence for this group. However, only one case in the intermediate group was classified as G_4. It was our opinion that G_1 and the intermediate group were highly interrelated, so the intermediate group was included in G_1, except for case 41, which was moved to G_4. The new group delimitation is shown in Figure 2.

A final SDA was then performed on the four newly defined groups. This analysis was done in three steps, including the three original variables in decreasing order of discrimination. In the first step, DNA was entered into the model; the value of Wilks' λ decreased to 0.311, which already indicated very good discrimination. The addition of the RNA in the second step and protein content in the third considerably increased the discriminant capacity of the final function, producing values of λ of 0.076 and 0.064, respectively.

Standardized coefficient values and the constant of each discriminant function are presented in Table 2. The first two functions explained as much as 99.6% of the total variance. Values of the canonical correlation coefficient were 0.9 and 0.8 for the first two canonical variables (Table 3), and 0.16 for the third.

The classification matrix obtained from the discriminant analysis (Table 3) was used to evaluate the accuracy of its discrimination. At the end of the analysis, 98.6% of the cases were correctly assigned into the groups. Only one case

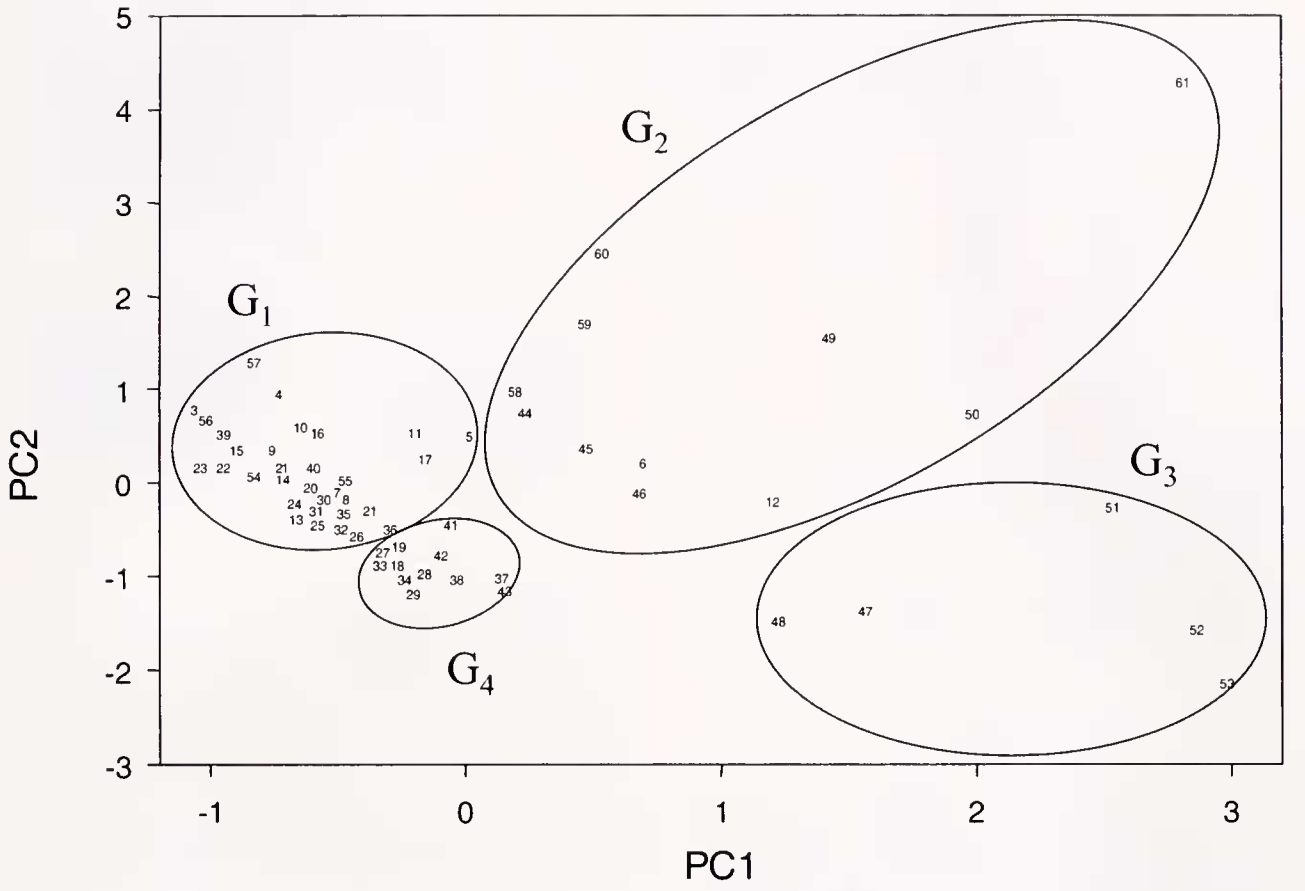

Figure 2. Plot of factor scores of the cases after principal components analysis with respect to first (PC_1) and second (PC_2) principal components, and the delimitation of the four final groups (G_1, G_2, G_3, and G_4). Delimitation of the final groups was obtained after three consecutive stepwise discrimination analyses.

from G_2 was misclassified in G_3. The coefficients and constants of the classification functions resulting from the SDA are presented in Table 4.

Thus, the groups were defined taking into consideration the results of the PCA and SDA, and also the nutritional background and the larval development of each case. The assignment of each case to each group is shown in the Appendix. The group definitions are explained in Table 5 and and illustrated in Figure 3.

More developed larvae were less resistant to starvation: after being starved, they needed a longer feeding period to recover their normal biochemical levels, and thus to be reclassified in the upper groups of the diagram in Figure 3.

All larvae that were never fed after mouth opening died 6.5 d after hatching.

Table 6 shows the mean values of RNA/DNA and DNA/dry weight for the four groups obtained through the previ-

Table 2

Standardized coefficients of each discriminant function of the final stepwise discriminant analysis (SDA) performed on the four groups obtained through principal component analysis and two previous SDAs

Variable	DF1	DF2	DF3
DNA	23.600	−3.848	18.962
RNA	−2.935	−3.911	2.996
Protein	−0.060	0.274	−0.537
Constant	−6.850	2.127	−2.723
Variance explained (%)	74.8	24.7	0.5
Canonical correlation	0.910	0.784	0.179

Table 3

Classification matrix of the final stepwise discriminant analysis (SDA) performed on the four groups obtained through principal components analysis and two previous SDAs

Group	% of correct classification*	G_1	G_2	G_3	G_4
G_1	100.0	33	0	0	0
G_2	90.9	0	10	0	1
G_3	100.0	0	0	5	0
G_4	100.0	0	0	0	12
Total	98.4	33	10	5	13

* A high value indicates that the groups are different and distinct.

Table 4

Classification functions resulting from the final stepwise discriminant analysis (SDA) performed on the four groups obtained through principal components analysis and two previous SDAs

Variable	G_1	G_2	G_3	G_4
DNA	183.9	274.6	345.6	224.6
RNA	0.3	0.3	−27.5	−8.1
Protein	−2.3	−3.2	−2.2	−2.3
Constant	−20.5	−52.8	−84.8	−30.7

The functions permit new cases to be classified into one of the four groups.

ous analysis and the results of the comparative ANOVA. RNA/DNA values are significantly different between G_1-G_3, G_1-G_4, G_2-G_3, and G_2-G_4. Therefore, this index is not useful for distinguishing between G_1-G_2, and G_3-G_4, since only two groups can be identified. DNA/dry weight is significantly different only between G_4 and the other three groups, and does not permit us to distinguish-between G_1, G_2, and G_3.

Discussion

RNA, DNA, and protein content have been used frequently as biochemical criteria to diagnose the nutritional condition in marine fish larvae (Bulow, 1970; Buckley, 1979b, 1984; Clemmesen, 1987, 1994; Cunha, 1991, 1996; Richard *et al.*, 1991; Takii *et al.*, 1994; Chícharo *et al.*, 1998). These variables alone or, more often, the indices RNA/DNA and DNA/dry weight have been used. However, we found the multivariate approach to be a more useful tool when using such biochemical data to assess nutritional condition in turbot larvae. The method was found to be more sensible than the two biochemical indices commonly used (RNA/DNA and DNA/dry weight), since it allowed the discrimination of larvae according to their real nutritional condition, and also provided additional information about the period when larvae were deprived of food. Larvae that never were fed or were deprived of food at an early stage (standard length < 4 mm or 5 d) could be distinguished from those fed and then deprived of food in a later stage of development. When the RNA/DNA index was used, similar values were obtained for larvae with different nutritional backgrounds and developmental stages. The DNA/dry weight index detected only larvae of group G4 as being in poor nutritional condition, appraising the larvae of the other three groups as being in similar good nutritional condition. We know that this is false because some of the larvae belonging to group G3 had been deprived of food for 72 h. Moreover, the appraisal of nutritional condition did not depend on a single variable, and so a multivariate analysis seems more appropriate from a theoretical point of view than a univariate approach. When the groups are unknown *a priori*, an ordination technique is preferable; the usual objective of ordination is to help generate hypotheses about the relationship between possible group composition and the underlying factors. Principal component analysis is one of the most commonly used ordination techniques. Once the

Table 5

Characteristics of larvae classified into the four groups obtained through principal components analysis according to their development and nutritional condition

Group	Age or length of larvae	Nutritional condition
G_1	Until day 5 or 4 mm	Sub-optimal • First days of development when fed adequately • First feeding delayed from 0 to 2 days • Starved less than one day
G_2	After day 5 or 4 mm	Optimal • Well fed • Starved less than one day
G_3	After day 5 or 4 mm	Very deficient • Starved more than one day
G_4	Until day 5 or 4 mm	Deficient and very deficient • First feeding delayed more than 2 days • Starved longer than one day

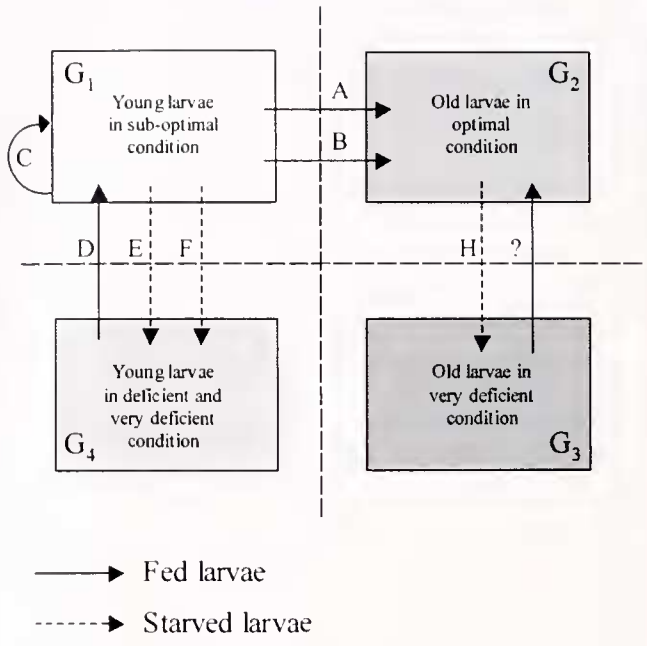

Figure 3. Theoretical scenario describing the evolution of larvae in relation to their developmental stage and nutritional condition, considering the four groups obtained from the principal components analysis (G_1, G_2, G_3, and G_4). (A) Larvae fed since mouth opening; (B) larvae whose first feeding was delayed for less than 12 h; (C) larvae whose first feeding was delayed for 30 h; (D) larvae whose first feeding was delayed for 54 h; (E) larvae in permanent starvation since mouth opening; (F) starved larvae that were previously fed for a period between 0 and 50 h; (H) starved larvae that were previously fed for more than 96 h.

Table 6

Mean values of RNA/DNA and DNA/dry weight indices of the four groups obtained through multivariate analysis

Group	Mean	SD	Max	Min	Tukey's HSD*			
RNA/DNA								
G1	3.10	0.55	4.36	2.09		G1	G2	G3
G2	3.69	0.58	4.57	2.90	G2	0.0516		
G3	2.02	0.54	2.92	1.59	G3	**0.0089**	**0.0002**	
G4	1.70	0.33	2.46	1.26	G4	**0.0002**	**0.0002**	0.7717
DNA/dry weight								
G1	12.53	1.88	16.28	8.96		G1	G2	G3
G2	10.48	1.10	12.67	8.55	G2	0.0571		
G3	12.78	1.65	14.26	10.94	G3	0.9964	0.2136	
G4	16.41	2.31	19.12	11.37	G4	**0.0002**	**0.0002**	**0.0156**

SD, standard deviation; Max, maximum value; min, Minimum value.

* Results of the Tukey unequal *n* honest significant difference test. Values shown are the *post hoc* probabilities (P). Statistically significant differences occur when $P < 0.001$ (bold values).

data have been classified into groups, the difference between those groups can be determined and a classification function can be estimated to ascertain the group to which a new sampled element belongs. Stepwise discriminant analysis may be, for many purposes, the most widely used classification technique, but it is seldom applied to biochemical data (Navarro *et al.*, 1995).

The multivariate analysis of the biochemical data shows a certain organization of the cases into groups. By examining the larval developmental stage and nutritional condition of each case and relating these two criteria with the biochemical analysis (RNA, DNA, and protein content), four groups were defined. This grouping aids in designing a scenario describing the evolution of the larvae in relation to their nutritional experience and development (Fig. 3). The first criterion, larval development, determines the horizontal position of the larvae in the chart. This determination is based mainly on DNA and protein content, as these two variables were the most important in the PC_1. Larvae in sub-optimal and optimal feeding conditions were classified in groups G_1 and G_2--the younger larvae in the former group and the older larvae in the latter.

The second criterion, nutritional condition, determines the vertical position of the larvae in the chart. This determination is based mainly on their RNA content, because this variable got the highest value in PC_2. Larvae in a deficient or very deficient nutritional condition—that is, those subjected to a long period of starvation and those whose first feeding was delayed for a long period of time (3 d)—had lower RNA content, leading to the discrimination of the inferior groups, G_3 and G_4. The lower RNA content of the larvae included in these groups determines their lower capacity for protein synthesis (Sutcliffe, 1965; Bulow, 1970; Millward, 1989). The protein content was also lower in these groups, but not the DNA content, demonstrating that starvation reduced the volume of the cells, not the number. In our opinion, the relationship between these two parameters, protein and DNA, sets the lower limit for starvation and determines larval death: the protein level cannot be lower than that required to maintain basic functions and minimal structures.

Larvae that were formerly classified in the upper groups were classified in the lower ones later, after starvation (Fig. 3). The opposite also occurred: those larvae in a deficient nutritional condition, belonging to group G_4, recovered their RNA levels after feeding and were later classified in group G_1—even those whose first feeding was delayed for 3 d. Owing to the insufficient number of larvae left after sampling, no further conclusions could be made about the point of irreversible starvation. But to the extent that we could sample, all larvae that were never fed after mouth opening died 6.5 d after hatching. This result is in accordance with Jones (1972), who observed 50% mortality in larvae 7 d after hatching; but his study was performed at 17.5 °C, whereas we were working at 20–21 °C. Therefore, the point of irreversible starvation for larvae that are never fed after mouth opening must be between days 3 and 6.5.

The analysis also indicates that, within each group, the most developed larvae are less resistant to starvation. In fact, early larvae that had been starved perform better after being fed for 6 h than those that were previously fed for 1.5 or 2.5 d. The probable explanation is that earlier larvae have larger yolk sac reserves, with a more positive energy balance. Moreover, the lower resistance of the more developed larvae might reflect higher metabolic activity rates and thus faster consumption of energy stores (Cunha, 1996).

The results presented in this paper demonstrate a new approach to determining the nutritional condition of reared fish larvae. They should however, be regarded with caution because larvae from different batches and geographic locations have different nucleic acid levels (Canino *et al.*, 1991). These levels are affected by temperature (Buckley, 1982, 1984; Buckley *et al.*, 1990, 1991; Canino, 1994) and may even show diel variations (Chícharo *et al.*, 1998). Moreover,

the experiments were restricted to the initial development of the larvae, including larvae younger than 12–13 d and smaller than 200 μg in dry weight. In spite of all these constraints, the type of analysis presented here seems promising. If the variables are controlled as discussed above, this classification technique seems very accurate. Current, biochemical analyses of nucleic acids are very precise, with the use of individual larvae, instead of pooled ones, recommended to increase precision. Thus this method can still be useful even if the experimental conditions are less drastic than those presented here, because of the wide range of nutritional conditions analyzed in this paper. However, since laboratory conditions do not always simulate natural conditions, the extrapolation of laboratory-derived values to the wild has often been questioned (Mackenzie *et al.*, 1990; Folkvord and Moksness, 1995). Nevertheless, the technique is straightforward, requiring only three input variables to characterize new samples of larvae, or individual larvae, with unknown nutritional and developmental backgrounds. RNA, DNA, and protein content of the new samples are input in the four classification functions, producing four different classification scores. The sample or individual is classified in the group whose function resulted in the highest score.

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Appendix

Initial conditions for turbot larvae in two experimental feeding treatments: nutritional background (time of starvation and feeding periods), age in day degrees, biochemical variables (dry weight, protein, RNA and DNA content) indices of these variables (RNA/DNA and DNA/dry weight). The group column indicates the group assignment in the analyses (see Results section). The case number refers to the number of each case in Figures 1 and 2.

Treatment A. Control (never fed) and experimental (initially starved and later provided with food) larvae

Starvation period (hours)	Feeding period after starvation (hours)	Day degrees	Dry weight (μg)	Protein (μg)	RNA (μg)	DNA (μg)	RNA/DNA	DNA/dry weight (×1000)	Group	Case number
	24	30.0	34.47	18.770	1.381	0.434	3.182	12.59	1	1
	48	50.0	37.67	20.030	1.325	0.419	3.162	11.12	1	2
	72	68.0	41.33	23.125	1.626	0.417	3.899	10.09	1	3
	96	87.0	52.67	26.645	2.171	0.537	4.043	10.20	1	4
	120	107.0	58.50	29.175	2.239	0.639	3.504	10.92	1	5
6	144	130.0	70.13	32.400	2.415	0.748	3.229	10.67	2	6
	24	30.0	32.60	17.140	1.223	0.439	2.786	13.47	1	7
	48	50.0	33.93	16.930	1.168	0.434	2.691	12.79	1	8
	72	68.0	36.47	19.690	1.553	0.453	3.428	12.42	1	9
	96	87.0	47.40	25.780	1.786	0.488	3.660	10.30	1	10
	120	107.0	53.43	28.940	2.062	0.583	3.537	10.91	1	11
12	144	130.0	63.53	32.855	2.363	0.805	2.935	12.67	2	12
	48	50.0	27.67	16.195	0.972	0.389	2.499	14.06	1	13
	72	68.0	33.27	19.050	1.193	0.402	2.968	12.08	1	14
	96	87.0	37.07	18.870	1.437	0.418	3.438	11.28	1	15
	120	107.0	44.87	22.400	1.861	0.512	3.635	11.41	1	16
30	144	130.0	53.88	25.845	1.841	0.562	3.276	10.43	1	17
	72	68.0	23.47	12.265	0.674	0.392	1.719	16.70	4	18
	96	87.0	26.27	13.960	0.819	0.411	1.993	15.65	4	19
	120	107.0	32.80	15.825	1.172	0.422	2.777	12.87	1	20
54	144	130.0	35.60	16.565	1.159	0.448	2.587	12.58	1	21

Control—starved larvae

	Starved during (hours)	Day degrees	Dry weight (μg)	Protein	RNA	DNA	RNA/DNA	DNA/dry weight (×1000)	Group	Case number
Newly hatched	0	0.0	32.13	15.43	1.250	0.391	3.197	12.17	1	22
	6	4.8	31.65	14.21	1.210	0.375	3.227	11.85	1	23
	8	9.6	30.67	14.55	1.189	0.428	2.778	13.96	1	24
	16	19.1	27.00	13.57	1.060	0.414	2.560	15.33	1	25
	30	25.0	25.40	13.79	0.831	0.397	2.093	15.63	1	26
	32	38.4	25.53	12.24	0.752	0.401	1.875	15.71	4	27
	54	45.0	22.33	12.21	0.611	0.401	1.524	17.96	4	28
	82	68.0	19.67	10.39	0.472	0.376	1.255	19.12	4	29

Treatment B. Control (continuously fed) and experimental (initially fed and later starved) larvae

Feeding period (hours)	Starvation period after feeding (hours)	Day degrees	Dry weight (μg)	Protein (μg)	RNA (μg)	DNA (μg)	RNA/DNA	DNA/dry weight (×1000)	Group	Case number
	6	9.6	30.00	14.42	1.209	0.431	2.805	14.37	1	30
	12	14.3	28.47	13.65	1.099	0.419	2.623	14.72	1	31
	24	24.0	26.73	12.81	0.930	0.409	2.274	15.30	1	32
	48	42.9	22.40	11.00	0.660	0.389	1.697	17.37	4	33
6	72	62.4	20.87	10.15	0.512	0.380	1.347	18.21	4	34
	6	28.8	30.80	15.23	1.146	0.435	2.634	14.12	1	35
	12	33.6	26.60	14.73	0.966	0.433	2.231	16.28	1	36
	24	42.9	26.00	13.77	0.807	0.468	1.724	18.00	4	37
30	48	62.4	23.27	12.53	0.632	0.419	1.508	18.01	4	38
	6	47.7	39.40	20.62	1.561	0.426	3.664	10.81	1	39
	12	54.4	38.87	20.16	1.426	0.456	3.127	11.73	1	40
	24	62.4	38.53	18.76	1.212	0.493	2.458	12.80	4	41
	48	81.9	38.00	15.97	0.815	0.432	1.887	11.37	4	42
50	72	103.5	27.40	13.84	0.625	0.440	1.420	16.06	4	43
	6	86.8	64.73	33.82	2.630	0.718	3.663	11.09	2	44
	12	91.6	62.33	33.24	2.369	0.710	3.337	11.39	2	45
	24	103.5	60.00	31.93	1.963	0.676	2.904	11.27	2	46
	48	125.7	50.20	25.63	1.384	0.716	1.933	14.26	3	47
96	72	147.5	44.20	21.97	0.984	0.617	1.595	13.96	3	48
	6	130.7	110.60	56.26	4.180	1.063	3.932	9.61	2	49
	12	135.8	104.20	54.67	3.688	1.063	3.469	10.20	2	50
	24	147.5	96.07	49.47	3.073	1.051	2.924	10.94	3	51
	48	170.0	83.87	41.27	1.876	0.925	2.028	11.03	3	52
150	72	191.0	64.40	35.64	1.420	0.883	1.608	13.71	3	53

Control—fed larvae

Fed during (hours)	Day degrees	Dry weight (μg)	Protein	RNA	DNA	RNA/DNA	DNA/dry weight (×1000)	Group	Case number
6	4.8	31.00	14.36	1.289	0.415	3.106	13.39	1	54
30	24.0	30.53	15.98	1.280	0.452	2.832	14.81	1	55
50	42.9	42.07	20.50	1.699	0.439	3.870	10.43	1	56
80	67.3	61.73	30.24	2.412	0.553	4.362	8.96	1	57
96	81.9	73.27	34.64	2.875	0.749	3.838	10.22	2	58
130	109.0	90.67	46.21	3.758	0.896	4.194	9.88	2	59
150	125.7	117.13	55.90	4.527	1.002	4.518	8.55	2	60
210	175.5	173.87	102.77	7.724	1.690	4.570	9.72	2	61